

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant: Kaare M. GAUTVIK et al.
Title: *Production of Human Parathyroid
Hormone From Microorganisms*
Appl. No.: 08/340,664
Filing Date: November 16, 1994
Examiner: L. Spector
Art Unit: 1647

DECLARATION OF KAARE M. GAUTVIK, M.D.

PURSUANT TO 37 C.F.R. § 1.132

Mail Stop NON-FEE AMENDMENT
Commissioner for Patents
PO Box 1450
Alexandria, Virginia 22313-1450

Sir:

I, KAARE M. GAUTVIK, declare as follows:

1. I am a co-inventor of the above-captioned application.
2. I am a citizen of Norway residing at Bregnevn 3, 0875 Oslo, Norway. I am fluent in English. My curriculum vitae is attached hereto as Exhibit A.
3. This Declaration supplements my Declaration submitted on August 11, 2004, regarding the synthetic hPTH(1-84) standard referred to on page 7, line 19, of the specification. The August 11 Declaration described an analysis on an SDS-PAGE gel of the five different synthetic PTH standards described in the specification, obtained from Peptide Institute Protein Research Foundation, Penninsula Laboratories, Sigma, and Bachem Fine Materials, as compared to recombinant hPTH(1-84) of the claimed invention. The SDS-

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PAGE gel showed that all of the five synthetic PTH compositions used as standards contained impurities.

4. The present Declaration describes how such impurities result in decreased PTH activity. Exemplary data for two of the synthetic PTH standards is described; from Sigma and Bachem Fine Materials.

I. Further Analysis of the Synthetic PTH Available From Bachem

A. The Bachem Synthetic PTH has Significant Impurities as Compared to Applicants' Claimed hPTH(1-84)

5. Recombinant hPTH (1-84) according to the invention was prepared. The recombinant hPTH(1-84) was produced as a secretory product by yeast *Saccharomyces cerevisiae* containing an expression vector encoding the entire cDNA for the hormone.

6. The hPTH(1-84) was purified by chromatography and HPLC.

7. The purified recombinant hPTH(1-84) was then compared to Bachem's synthetic PTH via silver-stained SDS-PAGE analysis. *See* Figure 1 (Exhibit B). The recombinant hPTH(1-84) was assessed to be more than 95% pure, as shown by the very thin band corresponding to the recombinant hPTH(1-84) on Figure 1 (Exhibit B).

8. In contrast, the Bachem synthetic PTH preparation contained significant small molecular weight impurities, as demonstrated by the blurry bottom edge of the gel band on Figure 1 corresponding to the Bachem synthetic PTH preparation (Exhibit B).

B. The Impurities Present in Bachem's Synthetic PTH Result in Significantly Reduced Activity as Compared to Applicants' Claimed hPTH(1-84)

9. The impurities present in the Bachem synthetic PTH preparation correlate with a significantly reduced biological activity as compared to recombinant hPTH(1-84).

10. For example, the ability of the Bachem synthetic PTH preparation to bind to the receptor and induce cAMP production in cultured cells transfected with the rat PTH

receptor was reduced by 40%, as compared to recombinant hPTH(1-84) from *E. coli* and yeast. See page 1262, Fig. 2 of Olstad et al., *Peptides*, 15(7):1261-1265 (1994) (Exhibit C).

11. Further, when tested *in vivo*, a similar reduced biological activity was observed for the Bachem synthetic PTH as compared to recombinant hPTH(1-84). This was determined by (a) measuring different PTHs' ability to increase blood calcium and (b) measuring changes in urinary cAMP, following injection of the two different PTH preparations to rats having their parathyroid hormone glands removed.

12. First, for measuring blood calcium elevation, blood samples from the rats were drawn at 0, 1, 2, 3, 4, 5, and 6 hours post administration. The biological activity of the Bachem synthetic PTH and recombinant hPTH(1-84) preparations was determined by measuring the difference between the concentration of calcium at the various time points compared to that in the baseline sample. See page 1262, Fig. 3 of Olstad et al. (Exhibit C).

13. The results showed that the Bachem synthetic PTH preparation had about a 50% lower ability to increase blood calcium levels, as compared to recombinant hPTH(1-84). See page 1262, Fig. 3 of Olstad et al. (Exhibit C).

14. Second, for measuring changes in urinary cAMP of rats having their parathyroid gland removed, urine was collected for 30 min. after administration of the Bachem synthetic PTH or the recombinant hPTH(1-84) preparation. See page 1263, Fig. 5 of Olstad et al. (Exhibit C).

15. The results showed that rats receiving the Bachem synthetic PTH preparation produced only about ½ or less cAMP in urine after injection, as compared to recombinant hPTH(1-84) preparation. See page 1263, Fig. 5 of Olstad et al. (Exhibit C).

16. These results demonstrate that the Bachem synthetic PTH has a significantly lower biological activity as compared to recombinant hPTH(1-84), clearly showing the non-authentic nature of the Bachem synthetic PTH preparation.

II. Further Analysis of the Synthetic PTH Available From Sigma

A. The Sigma Synthetic PTH has Significant Impurities as Compared to Applicants' Claimed hPTH

17. As another example, the Sigma synthetic PTH preparation is also impure.

18. For example, after two HPLC purifications steps, the recombinant hPTH(1-84) and the Sigma synthetic PTH elute as a peak showing a symmetrical profile. *See* Fig. 9B of U.S. Patent No. 5,420,242 (showing the recombinant h(PTH)(1-84) peak and the Sigma synthetic PTH peak) (Exhibit D) and Fig. 4C (recombinant hPTH(1-84) peak) and 4D (Sigma synthetic PTH peak), page 7341 of Hogset et al., *J. of Biol. Chem.*, 265(13):7338-7344 (1990) (Exhibit E).

19. However, an analytical gel electrophoresis with material from the two peaks after the second HPLC, carried out via silver staining of an SDS-PAGE gel, showed that the Sigma synthetic PTH preparation contained a significant high molecular weight impurity, in addition to low weight impurities shown under the PTH band. *See* Fig. 5D, page 7341 of Hogset et al. (Exhibit E).

B. The Impurities Present in Sigma's Synthetic PTH Result in Significantly Reduced Activity as Compared to Applicants' Claimed hPTH(1-84)

20. The impurities present in the Sigma synthetic PTH preparation correlate with a significantly reduced biological activity, as compared to recombinant hPTH(1-84). This was demonstrated in an adenylate cyclase assay of recombinant hPTH(1-84) and the Sigma synthetic PTH.


21. The relevant adenylate cyclase assay activity of the recombinant PTH(1-84) was shown to be significantly greater than that for the Sigma synthetic PTH. *See* Fig. 8, page 7342 of Hogset et al. (Exhibit E).

22. These results demonstrate that the Sigma synthetic PTH has a significantly lower biological activity as compared to recombinant hPTH(1-84).

Atty. Dkt. No. 016777/0310
Appln. No. 08/340,664

23. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated: 09.30.04


KAARE M. GAUTVIK, M.D.

**CURRICULUM VITAE
RELEVANT PUBLICATIONS
KAARE M. GAUTVIK, M.D., PH.D, CHIEF CONSULTANT**

Personal and marital status:

Name: Kaare M. Gautvik
 Home address: Bregnevn. 3, 0875 Oslo, Norway
 Business address: Institute of Medical Biochemistry, University of Oslo,
 P.O.Box 1112 Blindern, 0317 Oslo, Norway
 Telephones: 47-22851055 (work); 47-22235137 (home)
 Date and place of birth: 11th of December 1939 in Oslo.
 Social Security: No.: 111239.39311
 Married to: Vigdis Teig Gautvik, date of birth: 24th of March 1947
 Children: Lars Erlend Sakrisvold Gautvik, date of birth: 9th of January 1964
 Silja Marie Sakrisvold Gautvik, date of birth: 31th of March 1973, Ole Martin Teig Gautvik, date of birth: 21th of January 1982

Education and Clinical Specialities:

1. August 1958-June 64, Medical School at the University of Oslo.
2. 1967-69, Courses in mathematics involving geometry, statistics and mathematical analysis.
3. May 1970, Disputation for the medical doctor degree at the University of Oslo.
4. 1985, Specialist in clinical chemistry, and physiology and nuclear medicine.
5. 1986, Specialist in occupational health medicine.

Employment:

1. June 1964-June 1965, working at Tromsø University Hospital at medical and surgical departments.
2. July 1965 until December 1965, working as a general practitioner in Sjøvegan, Troms.
3. One year military service as a major in The Norwegian Air Force, working mainly at the Norwegian Institute for Aviation and Space Medicine.
4. From 1967, position as post-doctoral researcher at The Institute of Physiology, University of Oslo.
5. From September 1969, promoted to Assistant Professor at the University of Oslo, Institute of Physiology.
6. Leader and responsible for clinical and experimental endocrinological laboratory of Institute for Surgical Res., The National Hospital, Oslo, from 1973-89.
7. From 1976-1978, training as a specialist in clinical chemistry at the Norwegian Radium Hospital, Oslo.
8. From August 1983 appointed to full professor at the Institute of Medical Biochemistry, Medical Faculty, University of Oslo.
 (At the same time receiving offers of professor chairs at the Institute of Physiology, Medical Faculty and at the Institute of Physiology and Biochemistry, Faculty of Odontology).
9. From January 2002 employment as senior consultant at Department of Clinical Chemistry, Laboratory Division, Ullevål University Hospital and professor II at the University of Oslo.

Post-doctoral training abroad:

1. For three months in 1967, I worked as a lecturer at the Department of Physiology, Medical School, Birmingham University, England.
2. From August 1971 to July 1973 in receipt of Fogarty international post-doctoral fellowship at the Department of Pharmacology, Harvard School of Dental Medicine and Harvard Medical School.
3. 1980, 4 months Visiting Professor at Institute of Genetics, BMC, Uppsala University, Sweden.
4. 1995/96, 12 months Visiting Research Professor, The Scripps Research Institute, Dept. Mol. Biology, La Jolla, San Diego, USA.
5. 1997, 3 months Visiting Research Professor, The Scripps Research Institute, Dept. Mol. Biology, La Jolla, San Diego, USA.

Teaching responsibility:

1. One year teaching in aviation medicine for medical personnel and pilots.
2. I have given lectures and courses for medical students in following subjects:
 Haematology, kidney physiology, endocrinology, circulation, respiration and gastrointestinal physiology.
 From 1983 organized and given lectures and courses in molecular genetics at undergraduate and postgraduate level for students in medicine and sciences.
3. Organized interfaculty advanced courses within molecular endocrinology.
4. Lectures have been given in the following subjects at post-doctoral courses:
 Diseases of the thyroid gland (1973); Regulation of circulation in the gastrointestinal system (1973); Local hormones (1975); Endocrinology (annually from 1978); Tumour markers (1979); Calcium metabolism (annually from 1980); Ligands for peptide hormone-receptors, and Nuclein acid biochemistry (1984); TRH-receptors in prolactin-producing cells (1985). Molecular biology in medical research (yearly from 1983). Biochemical analysis on bone material (1991).
5. Invited lectures: Several places in the U.S., in Sweden, in Finland, and in England, as well as different places in Norway, a total of 37 as of 1995.

6. Chief organizer of post graduate scientific courses for the Medical Faculty at University of Oslo, 1986-1991.
7. Organizer of international scientific meetings within the frame of the following societies:
Acta Endocrinologica (European International Endocrine Society), The Scandinavian Physiology and Pharmacology Meetings, and the Norwegian Biochemical Society.
8. Introduced teaching in Molecular Biology for students at the Medical Faculty, Oslo.
9. Invited as Symposium Lecturer at international meetings in physiology and endocrinology and molecular biology as exemplified below:

Examples of specially invited symposium lectures:

1. February, 1990: "Production of recombinant human parathyroid hormone in E.coli and Saccharomyces cerevisiae and its potential use as drug in osteoporosis" by Kaare M. Gautvik, Eli Lilly Co., Indianapolis, USA, in a Biotechnology meeting.
2. June, 1990: Symposium lecturer and organizer: "Hormone receptors and cellular signal transduction. The XXII Nordic Congress in Clinical Chemistry, Trondheim, Norway.
3. July, 1990: Symposium lecturer: "Transmembrane signal systems involved in the regulation of prolactin secretion by hypothalamic peptide hormones in cultured pituitary cells. 2nd European Congress of Endocrinology, Ljubljana, Yugoslavia.
4. July, 1990: Symposium lecture: "Successful cloning and production of human parathyroid hormone (hPTH) in yeast as a secretory product". 5th European Congress on Biotechnology, Copenhagen, Denmark. (Unable to attend, and the lecture was held by cand.scient. Sjur Reppe).
5. August, 1990: Symposium lecture: "Processing and stability of human parathyroid hormone produced in E.coli and S.cerevisiae studied by in vitro mutagenesis". Workshop/Symposium on site-directed mutagenesis and protein engineering, Tromsø, Norway.
6. December, 1990: Invited by Professor Guo Hui-Yu, Guangzhou, China and Professor G.L. French, Hong Kong. Lecture entitled: "Expression of human parathyroid hormone as a secretory protein in prokaryotic and eukaryotic microorganisms". The Second International Conference on Medical Microbiology and Biotechnology Towards 2000, Guangzhou, China. (Did not attend as a protest against the punishment of the students rebellion in Peking).
7. January 1991: Invited to a Workshop by Dr. Stephen Green, Central Toxicology Laboratory, ICI, Alderly Park, Macclesfield SK10 4TJ, UK. Lecture entitled: "Synergistic effects of hormones and fatty acid on peroxisomal 8-oxydation, enzyme activities and mRNA levels".
8. January 1991: Invited to a Protein Engineering Meeting by Professor Ian Campbell, Biochemistry Department, Oxford University, Oxford, UK. Lecture entitled: "Cloning and expression of human parathyroid hormone in microorganisms".
9. Invited by Professors T.T. Chen, D.A. Powers, B. Cavari, Maryland Biotechnology Institute, Baltimore, MD, to held a symposium lecture at the 2nd International Marine Biotechnology Conference, October 13-16, 1991, Baltimore, Maryland, USA. (Could not attend).
10. May 1991: Invited by Professor Jan Carlstedt-Duke, Karolinska Institutet, Huddinge, to held a lecture in the seminar series "Novum Lectures in Cellular and Molecular Biology".
11. January 1992: Invited by Professor Armen H. Tashjian, Department of Molecular and Cellular Toxicology, Harvard School of Public Health and Department of Biological Chemistry on Molecular Pharmacology, Harvard Medical School, Boston, USA. Lecture entitled: "Use of antisense RNA in delineation of the mechanism of action of G-coupled hormones".
12. August 1993: Invited by Norwegian Society of Chartered Engineers, The Blindern Conference. Lecture entitled: "Experience from industrializing basal research".
13. November 1993: Invited by Karolinska sjukhuset, Stockholm, to held a lecture at "Graduate course in molecular endocrinology - a problem oriented approach". The lecture is entitled: "Region specific actions of parathyroid hormone in target tissues".
14. February 1994: Invited by GBF, Gesellschaft für Biotechnologische Forschung mbH, Braunschweig. Lecture entitled: "Expression of human parathyroid hormone in microorganisms and animal cells with special reference to signal sequence efficacy and intracellular modifications".
15. September 1994: Invited by Professor K. Dharmalingam, Department of Biotechnology, Madurai Kamaraj University, India, to held a lecture in the symposium "Gene expression systems", XVth IUBMB, New Delhi. Lecture entitled: "Expression of human parathyroid hormone in microorganisms, insect cells, mammalian cells and as a milk protein in transgenic mice".
16. November 1994: Invited by Professor A. Taschjian Jr., Harvard School and Public Health, Boston, to held a lecture in a seminar. Lecture entitled: "Certain structural and functional characteristics of the human TRH receptor cDNA and mapping of the gene".
17. February 11-13, 1995: Cairns, Australia, Workshop on "Animal models in the prevention and treatment of osteopenia".
18. February 1995: Int. Meeting of Calcified tissue research, Melbourne, Australia.
19. May 1996: Dublin University Program. "How to identify patients at risk for development of osteoporosis".
20. September 1996: Lecture at Scripps Research Institute, San Diego. "Unique hypothalamic specific mRNAs expressed by molecular subtraction hybridization".
21. September 1996: Invited seminar at the Astra Research Center, Montreal. "Cloning and expression of human polypeptide hormones with biomedical potential".
22. November 1996: Invited lecturer, The Norwegian Rheumatological Society, Oslo, "PTH (parathyroidea hormone) - The biochemical foundation for treatment of osteoporosis".
23. December 1996: Invited at Nordic Conference for Medical Treatment of Osteogenesis Imperfecta, Holmen Fjordhotel, Asker, Norway. "Characteristics of bone remodelling in patients with osteogenesis imperfecta".

24. January 1997: Invited lecturer at The Salgrenska Hospital in Sweden. "Characterization and functional analysis of novel hypothalamus genes as identified by directional tag subtraction".
25. 1998: Guest lecturer at Scripps Research Institute: "Hypothalamic calcium-calmodulin kinase-cloning and functional aspects".
26. February 1999: Only invited speaker from abroad at National Osteoporosis Congress in Rio de Janeiro, Brazil.
27. 2000: Lecture at NPS-Allelix company and Toronto University: "Parathyroid hormone regulated bone remodelling".
28. May 2000, Rio de Janeiro, Brazil. Member of the International Scientific Panel at the International Congress in Osteoporosis.

Honorary lectures and prizes:

1. In 1984 recipient of Professor Olav Torgersen's Prize and Memorial lecture. This prize and lecture was created by Professor Torgersen, the University of Oslo, who was one of the founders of the Society for Promotion of Cancer Research in Norway. Because he contributed with personal money, the prize and lecture had his name. The title of my lecture was: "The medullary thyroid carcinoma: a special type of familial and hormone producing cancer".
2. In 1984 I was given the international science prize called The Nordic Insulin Prize instituted by Professor Jacob E. Poulsen, who worked at the University of Copenhagen. This prize is given within endocrinology and the candidate is chosen from all the countries in Northern Europe. The money was donated by the Insulin Laboratory now the company Novo-Nordisk. At that time, only one Norwegian had previously received this prize. The prize was given for my studies regarding how hormones exerted their biological actions in target cells.
3. The Gunnerus Prize was given in 1986 by the Royal Society of Norwegian Scientists. This is a prize which is given to a scientist selected by this society for scientific merits obtained and again it was within the field of hormone structure and action.
4. In 1987 I received a prize within biotechnology created by the Research Park at the University of Oslo, which at that time was called the Innovation Centre, University of Oslo.
5. Novum Lectures in Cellular and Molecular Biology, which was associated with a scientific prize. Invited by Professor Jan-Åke Gustafsson at Novum, Huddinge, The Karolinska Institute, Sweden, in 1991. This was given based on my research with human parathyroid hormone in relation to its first cloning, expression and studies of actions.
6. Lectures at Harvard School of Public Health in Cellular and Molecular Biology in 1995, regarding cloning of hormone genes and their characterizations. Invited by Professor A.H. Tashjian Jr. at the Department of Molecular and Cellular Toxicology, Harvard School of Public Health and Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, USA.
7. Given a 3 years economical "Group Research Support of 0.8 M NOK per year from 1997" after national and international project evaluations.
8. Scientific prize (Abstract award) 1997, at the Endocrine Society "Cloning and Organization of the human TRH-receptor Gene".
9. Norwegian Endocrine Society Prize (S.Reppe) for "Sox4 – a PTH regulated transcription factor in bone".
10. Endocrine Society 1998: Poster Award: Cloning and regulation of the thyroliberin receptor gene.
11. Norwegian Endocrine Society prize 1998: Hormone regulated bone remodelling.
12. American Society for Bone and Mineral Research (ASBMR); 1999 Best Poster Award: The Transcription factor Sox-4 is expressed in developing cartilage and bone cells.

Consulting appointments:

1. Senior honorary consultant for NPS Biotechnology, Salt Lake City, Colorado, USA.
2. Consultant for Karolinska Institute, Stockholm, Sweden.

Referee activity:

I am or have been working as referee for the following international journals:

Endocrinology, J. Expl. Cell Res., Acta Physiol. Scand. (Kbh.), Eur. J. Endocrinol. (Acta Endocrinol. Scand. (Kbh.), Eur. J. Clin. Invest., Hormone Research, Acta Obstet. Gynecol. Scand., Journal of Endocrinological Investigation, Eur. J. Biochem., Experimental Cell Research, J. Biol. Chem.

Guidance for the academic doctor degree: Twenty three and 5 ongoing.

Supervision of postgraduate candidates: Presently three.

Supervision of students' main degrees: Nineteen.

Guest research workers from abroad: In my group we have had research visitors for periods of one to three years from Polen, Bulgaria, Sweden, Tyskland, Denmark, Iceland, India, Israel and USA.

Member of committees for the academic doctor degree in Norway and abroad: 15.

Member of advisory international/national committees for evaluation of professor positions: 14.

Honorary Societies: Member of the Norwegian National Academy of Science and Letters

Professional memberships: Norwegian Society of Biochemistry, Norwegian Society of Physiology, Norwegian Society of Endocrinology, Endocrine Society (USA), American Society for Bone and Mineral Research (USA)

Medical Faculty Responsibilities:

1. An elected member of the Medical Faculty 1987-1990.
2. A member of the Research Council at the Medical Faculty 1987-1990.
3. Chairman of Postgraduate Courses for Ph.D. and Dr.med. students at the Medical Faculty 1986-1991.
4. Member of the Institute Group Committee for the Preclinical Sciences from 1989 and present.
5. Member of the Medical Faculty's council for evaluation of postgraduate applications from 1989-1993.
6. Committee member of the Medical Faculty's Scientific Instrument Board, 1996-.
7. Committee for Medical Research collaboration and interaction between University of Oslo and the National Hospital, 2000-.

National- and International Research Council Responsibilities:

1. Leader of Chemical Peptide Synthesis Core facility 1984-1989.

2. Chairman for the Biotechnology Committee as a representative for Norwegian Research Council in an inter research council body, 1986-1989.
3. Member of The Norwegian Research Council for Science and the Humanities (NAVF) Committee for Physiology and Pharmacology, 1986-1989.
4. Development and function as responsible leader of the nationwide core facility for peptide synthesis, 1988-1991.
5. Member of the Premedical Institute Group Committee for Preclinical Sciences from 1989-2003.
6. Member of the International Scientific Board of Novo-Nordisk Research Committee, 1989-2001.
7. Member of the CIBA Foundation Scientific Advisory Panel from 1995 elected as representative from Norway, 1989-present.
8. Chairman of the Research Council in the Norwegian Association for Osteoporosis, 1993-2003.
9. Leader of DNA Sequencing Core facility of the Institute of Basic Science, 1999-present.
10. *Consultant and peer reviewer within Wallenberg Consortium North Technology Platforms DNA; SNP (single nucleotide polymorphism) Technologies and the Platform for Proteomics on behalf of the Board of the Wallenberg Consortium North, Stockholm, Sweden, 2001-2004.*
11. *Coordinator for Marie Curie Training Sites Fellowship No MCFH-200-00040 . "Oslo Doctoral Training Site for Diagnosis and Therapy of Osteoporosis"—2001-2005.*
12. *Coordinator of EU 6.Program STREP contract no 502941, "Molecular mechanisms of bone homeostasis" (OSTEOGENE). Eight partners in 5 countries- 2003-2006*

Awards and fellowships:

1967, 3 months, I worked as a lecturer at the Department of Physiology, Medical School, Birmingham University, England.

1971 to July 1973 in receipt of Fogarty international post-doctoral fellowship at the Department of Pharmacology, Harvard School of Dental Medicine and Harvard Medical School.

1980, 4 months Visiting Professor at Institute of Genetics, BMC, Uppsala University, Sweden.

1995/96, 12 months Visiting Research Professor, The Scripps Research Institute, Dept. Mol. Biology, La Jolla, San Diego, USA.

1997 Awarded for 3 years the Norwegian Research Council's Science Prize for outstanding research.

2001 Promoted by EU to become Oslo Doctoral Training Site for Diagnosis & Therapy Of Osteoporosis, received by a group consisting of scientists from University of Oslo, IMBA and the National Hospital.

2002 Member of CNS Molecular Biology group (leader Ivar Walaas) appointed as a "Research Theme Priority" at the Medical Faculty, 2002- 2007

2004 OSTEOGENE (Molecular mechanisms of bone homeostasis) project given the highest priority and the only selected for presentation within Health Region East 2004.

Other professional activities:

1. Founder of the Norwegian Association for Osteogenesis Imperfecta 1978 (Norsk Forening for Osteoporosis Imperfecta) together with Mrs. L. Myhre.
2. Founder of the Norwegian Association for Osteoporosis (Norsk Osteoporoseforening) 1993, together with Norwegian Women Public Health Association (NKS).

Patents:

I. Two U.S. patents, U.S. Patent No. 5.010.010 and No. 5.420.242 are held with international extensions in Europe, Japan, Canada, and Australia. In addition, three Divisional Applications are submitted to the U.S. Patent Office and elsewhere. These patents and patent applications in the different countries are covering specific methods related to the production, purification and characterization of PTH in microorganisms for the use in treatment of osteoporosis.

II. Inventor in patent application from Scripps Research Institute on: Novel hypothalamic mRNAs, the corresponding peptides and their functions.

Publications: More than 200 original articles published in internationally well reputed and refereed journals. Relevant articles are cited in relation to description of the research activities :

A BRIEF DESCRIPTION OF THE MAIN RESEARCH PROJECTS AND RELEVANT REFERENCES

A. STUDIES OF HYPOTHALAMIC SPECIFIC mRNAs OBTAINED BY A NOVEL SENSITIVE SUBTRACTION HYBRIDIZATION PROCEDURE

The results so far from our refinement and usage of a powerful and highly sensitive novel subtractive nucleic acid hybridization method have been successful. The generated hypothalamic subtraction library appears to give a specific and comprehensive representation of mRNAs that are not present in other brain areas as hippocampus and cerebellum. We have so far described several novel peptides: hypocretin (the cause of Narcolepsy) and several very interesting peptides, e.g. novel CaM kinase (see list of references). Another CNS peptide is somatostatin-like, called cortistatin, structure similarity with somatostatin; P25 and Vat 1, two uniquely expressed peptides in distinct regions of the brain.

1. Gautvik, K.M., de Lecea, Luis, Gautvik, V.T., Danielson, P.E., Tranque, P., Dopazo, A., Bloom, F.E. and Sutcliffe, J.G. Overview of the most prevalent hypothalamus-specific mRNAs identified by directional tag PCR subtraction.

Proc. Natl. Acad. Sci. USA (PNAS) 93: 8733-8738, 1996.

2. de Lecea, L., Criado, J.R., Prospero-Carcia, O., Gautvik, K.M., Schweitzer, P., Danielson, P.E., Dunlop, C.L.M., Siggin G.R., Henriksen, S.J. and Sutcliffe, J.G. A cortical neuropeptide with neuronal depressant and sleep-modulating properties. Nature 381: 242-245, 1996.

3. de Lecea, L., Kilduff, T.S., Peyron, C., Gao, X-B., Foye, P.E., Danielson, P.E., Fukuhara, C., Battenberg, E.L.F., Gautvik, V.T., Bartlett II, F.S., Frankel, W.N., Van den Pol, A.N., Bloom, F.E., Gautvik, K.M. and Sutcliffe, J.G. The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proc. Natl. Acad. Sci. USA (PNAS) 95: 322-327, 1998.

B. ISOLATION AND CHARACTERIZATION OF mRNAs SPECIFIC FOR THE BONE CELL PHENOTYPE OBTAINED BY SUBTRACTION HYBRIDIZATION

By using the same novel subtractive hybridization procedure as employed and described above, we have generated a subtracted cDNA library using the osteosarcoma phenotype cDNA library as made from three different human osteosarcoma cells from which is subtracted the cDNA library obtained from normal human osteoblasts. The subtraction is performed by using cDNA from osteosarcoma cells minus RNA transcribed from the corresponding cDNA library of the normal osteoblast. These are experiments in progress and we are about to describe individual clones obtained from a subtracted library of about 400.000 independent colonies. The aim of this study is to identify those mRNAs which are overexpressed or lacking in the osteosarcoma phenotype and compile these results in order to have a greater understanding regarding how a normal cell is transformed into this tumor type (Olstad et al. ,2003)

C. ISOLATION AND CHARACTERIZATION OF mRNAs SPECIFIC FOR PARATHYROID HORMONE GENE ACTIVATION IN BONE CELLS ("Bone anabolic genes")

Parathyroid hormone is the most important physiological regulator of bone formation. This hormone therefore is assumed to represent an important drug in the prevention and especially treatment of postmenopausal osteoporosis. However, as a succession of our previous work regarding the studies of this hormone, we have continued to search for a complete overview of all gene products that parathyroid hormone is stimulating in bone cells in order to isolate the mRNAs and corresponding proteins which may be of central importance for the development of osteoporosis - or which may be called "the genes for osteoporosis". Again by using the same molecular subtraction method as described in Chapter II, C, we use this time parathyroid stimulated normal bone cells cDNA library minus RNA transcribed from generated libraries of normal bone cells. This work is almost completed in a highly successful manner. We have isolated more than 40 genes which are involved in PTH anabolic action in bone, and among those we are searching for the gene(s) causing postmenopausal osteoporosis.

D. As a complementation an to the activities described above, we have embarked on defining the bone phenotype in female and male osteoporosis within the context of the EU project OSTEOGENE (see above). About 100 patients and controls will have bone biopsies which will be prepared and analysed for their global gene expression and differences at the micro- and ultrastructural level. I am the coordinator of this activity including 5 countries and where Oslo university and three hospitals(Ullevål university hospital, the National Hospital and Lovisenberg hospital are working closely together. This is a direct consequence and follow up of previous research representing patient related basic and translational science aiming to solve the mechanisms of osteoporosis, the most common disease in women of 50 yrs of age.

THE MAIN RESEARCH ACTIVITIES DURING THE LAST 8 YEARS AND FUTURE SCIENTIFIC ENGAGEMENT:

I. PARATHYROID HORMONE (PTH) AND PARATHYROID HORMONE RELATED PROTEIN (PTHrP)

The aim for this work was to produce:

- i) Recombinant parathyroid hormone for structure activity studies in relation to bone cell activation.
- ii) Study intracellular processing and trafficking of these hormones and to compare signal sequence efficacy in different host expression systems.

We were the first in the world to clone and produce full-length human recombinant parathyroid hormone in mg quantities. For this work we developed gene constructs, vector modifications, fermentation technological improvements as well as complete methods for down-stream technology. The final product is PTH identical and more than 99% pure and has shown full chemical, biochemical and biological identity with the intact hormone. These results are written in the following articles that are printed.

We have also been as indicated by the list of references below, the first in the world to express secreted human parathyroid hormone in mammalian cells as well as a secretory milk product in transgenic mice. In addition, we have been the first to develop full-length PTH polypeptides with agonist and antagonist functions.

1. Høget, A., Blingsmo, O.R., Gautvik, V.T., Sæther, O., Jacobsen, P.B., Gordeladze, J.O., Alestrøm, P. and Gautvik, K.M. Expression of human parathyroid hormone in *Escherichia coli*. BBRC 166: 50-60, 1990.
2. Gabrielsen, O.S., Reppe, S., Sletten, K., Øyen, T.B., Sæther, O., Høget, A., Blingsmo, O.R., Gautvik, V.T., Gordeladze, J.O., Alestrøm, P. and Gautvik, K.M. Expression and secretion of human parathyroid hormone in *Saccharomyces cerevisiae*. Gene 90(2): 255-262, 1990.
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In this regard we have received acceptance for an international patent on gene constructions, plasmids, the process and the down-stream technology. In the further work we have by using *in vitro* mutagenesis, created full length parathyroid hormone agonist which has shown to be protease resistant and have interesting biological actions regarding mobilization of calcium from bone.

Both the intact hormone as well as the agonist will represent important medical drugs for use in diagnostics as well as represent a potential drug for treatment of various diseases.

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Saccharomyces cerevisiae. Identification of New motif for O-glycosylation. Eur. J. Biochem. 205: 311-319, 1992.

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Recently we have expressed the first known full length antagonist for hPTH, a long sought for molecule of considerable clinical interest. The compound has a binding KD which is 2-4 times less than the natural hormone, but shows a more than 100-fold reduced biological activity.

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Item 3 a
PRODUCT DATA SHEET (from Peninsula Laboratories) 29.6.90

Product: Recombinant human parathyroid hormone 1-84 amino acid peptide from yeast (Saccharomyces cerevisiae).

Cat.nr.: Lot nr.:

Quantity: mg PTH, freeze dried in 30 per cent acetonitril.

Source of the product.

The recombinant human parathyroid hormone is produced as a secretory product by yeast Saccharomyces cerevisiae containing an expression vector encoding the entire cDNA for the hormone (1,2).

Purification and purity.

The hormone was purified by chromatography, and HPLC. The final product was assessed to be more than 95% pure as judged by silver-stained PAGE analysis (Fig. 1). The product was furthermore validated by having a correct molecular weight and mass, N-terminal sequence and amino acid composition.

Biological activity was judged by adenylyl cyclase (AC) measurements using UMR osteosarcoma cell membranes and calcium mobilization from mouse bone organ culture. The recombinant product was found to be equal in potency to the synthetic 1-84 hormone.

Immunoblots showed that the recombinant PTH had the same activity towards N-terminal and mid-region specific antisera as chemically synthesized (1,2).

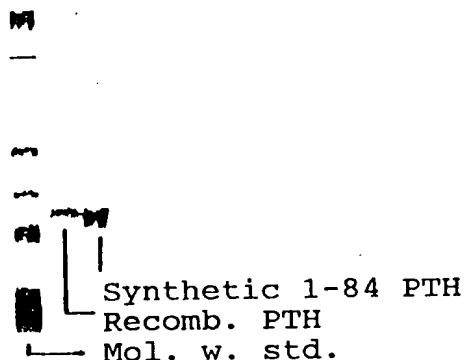


Fig. 1 (ref 1)

Storage and stability:

Stable for at least 6 months at -20°C. Avoid freezing and thawing, especially when dissolved.

Dissolvment of the product for usage:

The product is most stable when dissolved in acid solution eg. acetic acid or HCl, pH 2-4. The peptide is sticky and adhere easily to surfaces.

Referanser:

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Differences in Binding Affinities of Human PTH(1–84) Do Not Alter Biological Potency: A Comparison Between Chemically Synthesized Hormone, Natural and Mutant Forms

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OLSTAD, O. K., N. E. MORRISON, R. JEMTLAND, H. JÜPPNER, G. V. SEGRE and K. M. GAUTVIK. *Differences in binding affinities of human PTH(1–84) do not alter biological potency: A comparison between chemically synthesized hormone, natural and mutant forms.* PEPTIDES 15(7) 1261–1265, 1994.—The purpose of this study was to evaluate receptor binding affinities and biological properties in vitro and in vivo of various recombinant hPTH(1–84) forms representing the natural hormone and a mutagenized hPTH form, [Gln²⁶]hPTH(1–84) (QPTH), after expression in *E. coli* and *Saccharomyces cerevisiae*. In LLC-PK₁ cells stably transformed with the rat PTH/PTHrP receptor, chemically synthesized hPTH(1–84) and QPTH showed a reduced binding affinity (apparent K_d 18 and 23 nM, respectively) than the recombinant, hPTH(1–84) (apparent K_d 9.5 nM). All recombinant hPTH forms showed a similar potency to stimulate cellular cAMP production (EC_{50} 1.5 nM) and significantly better than chemically synthesized hPTH (EC_{50} 5.7 nM). All hormone forms showed an about equipotent activity in causing elevation in serum calcium, increased excretion of urine phosphate, and cAMP. Thus, the natural recombinant PTH forms showed higher binding affinities and adenylate cyclase activation potencies in LLC-PK₁ cells, but the reduced receptor binding affinity exerted by QPTH did not transcend differences in cAMP generation and in vivo biological activities.

Recombinant parathyroid hormones Recombinant PTH/PTHrP receptor cAMP response Rats

PARATHYROID hormone is the principle regulator of calcium homeostasis in humans and has been advanced as an anabolic drug against postmenopausal osteoporosis (22,25). The hormone, which is produced in the mammalian parathyroid glands, is synthesized as an 115 amino acid precursor that is processed to the mature hormone of 84 amino acids (21). The information required for high-affinity binding of PTH to its receptor in bone and kidney cells is contained within the biologically active 1–34 region (20). The amino-terminus of PTH is essential for triggering the adenylate cyclase response pathway (8,26), but it also contributes modestly to receptor binding affinity. In addition to a nearly complete loss of cAMP agonism, the deletion of residues 1–6 is accompanied by an approximately 100-fold decrease in receptor binding affinity (7,10,18,24). The major component of PTH receptor binding affinity, however, appears to be determined by residues 28–34. Deletion of these residues causes at least a 1000-fold reduction in binding affinity (18). Furthermore,

PTH(25–34) displays weak, but detectable, receptor binding affinity ($K_d \approx 100 \mu M$) (18). In comparison, no evidence for receptor interaction has been obtained for amino-terminal fragments shorter than PTH(1–27) (24,26). Based on these observations, the 25–34 region has been called the hormone's principal receptor binding domain (18).

We have previously reported production of hPTH(1–84) in yeast (5), and the α -factor expression system is a well-characterized, commonly used strategy for expression of foreign proteins by the yeast *Saccharomyces cerevisiae* (3,27,29). The mating factor alpha (MF α) leader sequence is cleaved off sequentially by the KEX-2 endopeptidase and then by an amino peptidase STE13, leaving a correct N-terminal after guiding the recombinant protein through the secretory pathway (11). In the expression plasmid p α UXPTH-2, the MF α promoter, signal sequence, and termination signal were employed. The secreted hormone was purified from medium to more than 95% homo-

¹ Requests for reprints should be addressed to O. K. Olstad.

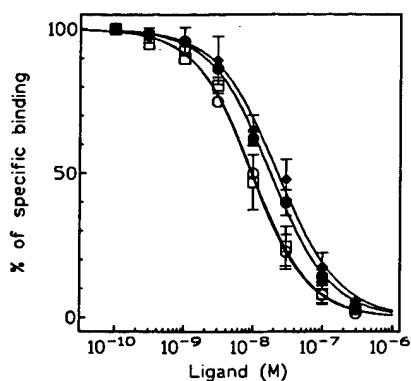


FIG. 1. Inhibition of radiolabeled [Tyr³⁶]chickenPTHrP(1-36)amide by different hPTHs. Recombinant hPTH(1-84) produced in *E. coli* (○), recombinant hPTH(1-84) produced in *Saccharomyces cerevisiae* (□), [Gln²⁶]hPTH(1-84) (QPTH) produced in *Saccharomyces cerevisiae* (◆), and chemically synthesized hPTH(1-84) (●) were tested in radioreceptor assay using LLC-PK₁ cells transfected with the rat PTH/PTHrP receptor. The data represent the mean \pm SD of at least two independent experiments, each performed in triplicate.

geneity, characterized chemically, and shown to represent the natural hormone (5,19). In addition to the intact hormone, an aberrant KEX-2 cleavage occurring at an internal site (5) after two consecutive basic amino acids in the hPTH sequence -Arg²⁵-Lys²⁶↓Lys²⁷-resulted in part fragmentation of the hormone. To improve the yield of hPTH, and to avoid internal degradation, a point mutation was introduced into the gene, changing Lys in position 26 to Gln (Q) (23). The resulting agonist, [Gln²⁶]hPTH(1-84), called QPTH, was tested together with recombinant hPTH(1-84) produced in *Saccharomyces cerevisiae* and compared with chemically synthesized hPTH(1-84) in certain biochemical and biological tests.

We have also produced full-length hPTH in *E. coli* as a secretory product employing the *Staphylococcus aureus* protein A signal and regulatory sequences (9). After purification from

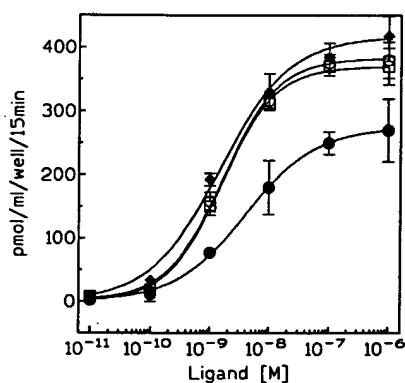


FIG. 2. Stimulation of cAMP by different hPTHs. Accumulation of intracellular cAMP in LLC-PK₁ cells transfected with the rat PTH/PTHrP receptor stimulated (15 min, 37°C) with recombinant hPTH(1-84) produced in *E. coli* (○), recombinant hPTH(1-84) produced in *Saccharomyces cerevisiae* (□), [Gln²⁶]hPTH(1-84) (QPTH) produced in *Saccharomyces cerevisiae* (◆), and chemically synthesized hPTH(1-84) (●). The data represent the mean \pm SD of two independent experiments, each performed in duplicate.

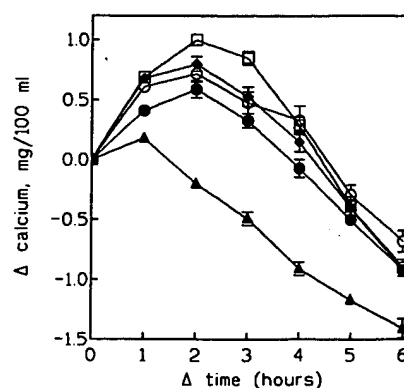


FIG. 3. Induction of hypercalcemia by different hPTHs. Parathyroidectomized male Wistar rats were administered different forms of hPTH (2.0 μ g of the recombinant hPTHs, 2.7 μ g of the chemically synthesized hPTH) as described in the Method section. The stimulating agents were recombinant hPTH(1-84) produced in *E. coli* (○), recombinant hPTH(1-84) produced in *Saccharomyces cerevisiae* (□), [Gln²⁶]hPTH(1-84) (QPTH) produced in *Saccharomyces cerevisiae* (◆), and chemically synthesized hPTH(1-84) (●). Control (▲). Blood samples were drawn at 0, 1, 2, 3, 4, 5, and 6 h after injection of PTH. The results are reported as the difference between the amount of calcium in the blood at the various time points, subtracting out the amount of calcium in the baseline sample (delta values). The data represents the mean \pm SEM ($n = 6$).

medium and chemical characterization, this recombinant form was also included in the biochemical and biological characterizations.

LLC-PK₁ cells (porcine renal epithelial cells) stably transfected with the cDNA for the rat PTH/PTHrP receptor (4) were used for the receptor binding studies and cAMP responsiveness; rats

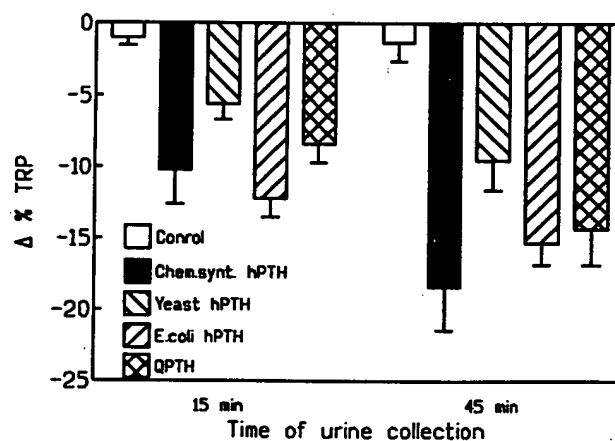


FIG. 4. Urinary excretion of phosphate. Parathyroidectomized male Wistar rats were administered different forms of PTH (2.0 μ g of the recombinant hPTHs, 2.7 μ g of the chemically synthesized hPTH) as described in the Method section. Urine was collected for two periods: 0-30 and 30-60 min after administration of PTH. The excretion of phosphate is expressed as the percent tubular reabsorption of phosphate (% TRP) and is calculated by the formula: $(1 - \text{phosphate clearance} / \text{creatinine clearance}) \times 100$. The result is reported as a change in % TRP related to the zero control level, and a decrease represents a greater amount of phosphate excreted in the urine. The data represents the mean \pm SEM ($n = 6$).

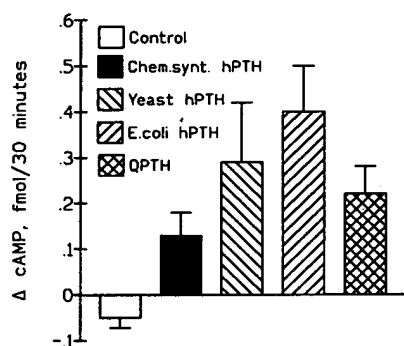


FIG. 5. Changes in urinary cAMP after administration of PTH. Parathyroidectomized male Wistar rats were administered different forms of PTH (2.0 μ g of the recombinant hPTHs, 2.7 μ g of chemically synthesized hPTH) as described in the Method section. Urine was collected for 30 min after administration of PTH. The excretion of cAMP is reported as a change in cAMP concentration related to the zero control level. The data represents the mean \pm SEM ($n = 6$).

were used for measurements of the hypercalcemic response, urine phosphate, and cAMP.

METHOD

Chemically synthesized hPTH(1-84) was purchased from Bachem Fine Chemicals (Torrance, CA) and [Tyr³⁶]chicken-PTHrP(1-36)-NH₂ for radioiodination was from Peninsula Laboratories. The production, purification, and chemical characterization of recombinant PTHs have been described previously (5,9,19,23). Peptide concentrations were determined by amino acid analysis. The blood and urine samples were analyzed for calcium, phosphate, protein, and creatinine on the Cobas Bio Autoanalyzer. cAMP was analyzed using a commercial radioimmunoassay kit from Amersham. All reagents were of highest purity available.

Radioreceptor Assay

LLC-PK₁ cells expressing the rat PTH/PTHrP receptor (4) were plated in 24-well plates. The cells were incubated with [¹²⁵I]-labeled [Tyr³⁶]chickenPTHrP(1-36)-NH₂ (100,000 cpm per well/0.5 ml) in the presence or absence of competing ligands at 15°C for 4 h, using a Tris-based binding buffer (50 mM Tris-HCl), pH 7.7, 100 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 5% heat-inactivated horse serum, 0.5% heat-inactivated fetal calf serum as described (28). The competing ligands included chemically synthesized hPTH(1-84) from Bachem, recombinant hPTH(1-84) expressed in *E. coli* (9), recombinant hPTH(1-84) expressed in yeast (5,19), and recombinant QPTH expressed in yeast (23). Techniques used for radioiodination of PTHrP analogue have been reported (12,13). PTH and PTHrP bind to and activate PTH receptors in bone and kidney in an indistinguishable manner (12,14). [¹²⁵I][Tyr³⁶]chickenPTHrP(1-36)-NH₂ was used as radioligand because of lower nonspecific binding (less than 5% of total binding) (14) compared to [¹²⁵I][Nle^{8,18}Tyr³⁴]bovine-PTH(1-34)-NH₂, which gave 10–15% nonspecific binding (28).

Intracellular cAMP Measurements

For measurements of intracellular cAMP, LLC-PK₁ cells (4) expressing the rat PTH/PTHrP receptor were plated in 24-well plates (50,000 cells/well) and grown to confluence for 3 days

(about 250,000 cells/well). The cells were placed on ice, rinsed once with 1 ml of cold Dulbecco's modified Eagle's medium containing 2 mM 3-isobutyl-1-methylxanthine, and 0.1% bovine serum albumin. Medium (0.5 ml) with or without PTH was added and cells were transferred to a 37°C water bath for incubation in 15 min. Then the cells were rinsed once with 0.5 ml phosphate-buffered saline and immediately frozen on liquid nitrogen. Intracellular cAMP was measured by a radioimmunoassay kit from Amersham, after lysing the cells with 1 ml of 0.05 N HCl.

Hypercalcemic Assay

Male Wistar rats (150–200 g) were parathyroidectomized using electrocautery 18 h prior to the start of the experiment. Preliminary experiments showed that this was a reliable way to obtain complete removal of parathyroid gland activity because plasma calcium fell linearly as a function of time, as also indicated by the control group in Fig. 3. Moreover, individual rats showed small variations in the results. The parathyroid glands were removed for two reasons. One, to eliminate the endogenous production of the hormone, and two, to make the animals more sensitive to exogenous hormone. The increase in sensitivity is assumed to be due to the upregulation of PTH receptors in target organs (16). Thus, it has previously been shown that tubular membranes prepared from parathyroidectomized rats reveal a higher binding of [³H]hPTH(1-34) and higher maximum stimulation of PTH-stimulated adenylate cyclase compared to control animals (16). It has also been shown (28) that downregulation of PTH receptors in ROS 17/2 cells occurs when the cells are exposed to PTH concentrations near hormonal physiological doses. More than 50% of the of the PTH-stimulated adenylate cyclase activity was recovered within 24 h after desensitization.

The animals were fasted overnight, and anesthetized the next day using hypnorm dormicum (0.2 ml/rat). The carotid artery was cannulated using polyethylene-50 tubing. The cannula was connected to a syringe containing Ringers acetate, 4% bovine serum albumin, 25 units heparin/ml. Five minutes after injection of 200 μ l of the heparinized Ringers acetate, a baseline blood sample was drawn (300 μ l). The animals were tracheotomized to prevent respiratory failure due to damage to the recurrent laryngeal nerve running through the thyroid gland. The PTH agonist was then injected SC in a volume of 200 μ l. All agonists were dissolved into 100 μ l of 0.01 N acetic acid. The test agents included:

1. vehicle, 0.001 N acetic acid, 1% bovine serum albumin (control),
2. chemically synthesized human PTH(1-84), 2.7 μ g/rat (chem. synt. hPTH),
3. recombinant human PTH(1-84) from yeast, 2.0 μ g/rat (yeast hPTH),
4. recombinant human PTH(1-84) from *E. coli*, 2.0 μ g/rat (*E. coli* hPTH),
5. recombinant [Gln²⁶]hPTH(1-84) from yeast, 2.0 μ g/rat (QPTH).

Due to the reduced receptor binding affinity and cAMP stimulation in the in vitro assays below, the chemically synthesized hPTH concentration was used at 2.7 μ g/rat.

After dissolving in acetic acid, the agents were brought up in 900 μ l of Ringers acetate containing 1% bovine serum albumin. Blood samples were drawn at 1, 2, 3, 4, 5, and 6 h after injection of hPTH or agonist. The rats were reheparinized 5 min before

drawing each blood sample using 200 μ l of the heparinized Ringers solution.

All forms of hPTH were analyzed and quantified by amino acid analysis before administration to the rats.

The blood samples were centrifuged in a clinical centrifuge for 10 min, then the plasma was analyzed for calcium using a Cobas Autoanalyzer.

Urine Analysis

Male Wistar rats (150–200 g) were parathyroidectomized using electrocautery 18 h prior to the start of the experiment. The animals were fasted overnight, and anesthetized the next day using hypnorm dromicum (0.2 ml/rat). The carotid artery and the jugular vein were cannulated using polyethylene-50 tubing. The cannula was connected to a syringe containing Ringers acetate, 4% bovine serum albumin, 25 units heparin/ml. The bladder was catheterized using PE-200 tubing.

The carotid artery was cannulated for the collection of blood samples, and the jugular vein was cannulated for the purpose of injecting the hormones, and for a slow infusion for the purpose of volume loading the rats to increase the urine output. The rats were infused with Ringers acetate, 4% bovine serum albumin at the rate of 3 ml/h. The infusion was run for 2 h before the start of the experiment to equilibrate the animals.

After the 2-h equilibration period, a baseline urine collection was made for 30 min, with a midpoint arterial blood sample drawn at 15 min. At the end of the baseline urine collection, the PTH was injected IV, and a new 30-min urine collection was started. Again, a midpoint blood sample was taken 15 min into the urine collection. A final 30-min urine collection was made from 30–60 min after the injection of PTH, with the midpoint blood collection made at 45 min after PTH injection.

The excretion of phosphate is expressed as the percent tubular reabsorption of phosphate (% TRP). The % TRP is calculated by the formula: $(1 - \text{phosphate clearance/creatinine clearance}) \times 100$. A decrease of % TRP represents a greater amount of phosphate excreted in the urine. The creatinine clearance did not change in any of the treatment groups. It was not expected to change, and was only measured to calculate the % TRP.

Statistical Analyses

A two-sample *t*-test was used comparing mean values of control and treated groups of animals (2).

RESULTS

Radioreceptor Binding Studies and Intracellular cAMP Measurements

Binding of the different hPTH forms is shown in terms of displacement curves using the [125 I][Tyr 36]chicken PTHrP(1–36)-NH $_2$ as radioligand and LLC-PK $_1$ cells permanently transfected with the rat PTH/PTHrP receptor.

The chemically synthesized hPTH and QPTH had calculated binding affinities with K_d of 18 nM (95% confidence interval: 16.1–20.0 nM) and 23 nM (95% confidence interval: 19.0–27.2 nM), respectively (Fig. 1). The natural recombinant hPTH(1–84) forms from *Saccharomyces cerevisiae* and *E. coli* had a similar but significantly lower apparent K_d of 9.5 nM (95% confidence interval: 8.7–10.4 nM) (Fig. 1). In spite of these differences in receptor binding affinities, all the recombinant hormones had equal ability to stimulate intracellular cAMP accumulation (EC_{50} about 1.5 nM, 95% confidence interval: 1.0–2.2 nM) (Fig. 2). In contrast, the synthetic hPTH showed a significant reduced potency to stimulate cAMP production with EC_{50} of 5.7 nM

(95% confidence interval: 3.4–9.6 nM) on a molar basis, and a reduced maximal response.

Hypercalcemic Assay

After parathyroidectomy, the control calcium concentration fell linearly 1 h after the operation (about 0.75 mg %/h) (Fig. 3). The chemically synthesized hPTH was injected in a dose of 2.7 μ g/rat compared to 2.0 μ g/rat employed for the other hPTH species due to the reduced receptor binding affinity and cAMP stimulation of the chemically synthesized hPTH. These concentrations were chosen on the basis of preliminary experiments using a range of different doses and were selected because they gave a healthy hypercalcemic response and no observable side effects (e.g., unaffected rectal temperature). The hypercalcemic response of the chemically synthesized preparation was somewhat lower than for yeast hPTH, but almost similar to the other recombinant hPTHs. From these experiments it appeared that the declining parts of the curves were similar and like the slope of the control curve (Fig. 3).

Tubular Reabsorption of Phosphate

The percent tubular reabsorption of phosphate (% TRP) was calculated on basis of urine creatinine values and showed a strong and significant ($p < 0.01$) reduction after injection of the different PTH forms, and the potencies were similar. This effect was already observed 15 min after injection, and was then close to or at its maximum (Fig. 4). (For calculation of % TRP, the Method section.)

Measurements of Cyclic Adenosine Monophosphate

The changes in the cAMP content of the urine after administration of PTH was somewhat variable, with the chemically synthesized hPTH showing the smallest effect. However, all forms of PTH responded in a similar fashion. Therefore, there is no principal difference between the preparations of PTH in terms of their stimulation of cAMP release into the urine (Fig. 5).

DISCUSSION

Structural analysis of PTH indicates that PTH(19–34) fragment contains substantial helical structure (17) and the residues 17–28 form an α -helix (15). This assumption has been confirmed (6), showing that mutations of the hydrophobic residues Leu 24 , Leu 28 , and Val 31 in hPTH are critical for optimal PTH activity, in contrast to most mutations of the polar residues (i.e., Lys 26 , Lys 27 , Gln 29 , Asp 30 , and His 32).

We have previously showed that QPTH is fully active in assays of adenylate cyclase, and this observation has been confirmed (6). Also in bone resorption studies using mouse calvaria (23), the QPTH was equally potent compared with the natural hormone. Biotinylation of Lys 26 or Lys 27 of [Nle 8,18 , Tyr 34]bPTH(1–34) has no effect on binding affinity (1), but substitutions as Lys $^{26} \rightarrow$ Glu and Lys $^{26} \rightarrow$ Thr causes partial reduction in cAMP production by PTH stimulation (6).

Interestingly, the substitution in QPTH, Lys $^{26} \rightarrow$ Gln, lowers the hormone's affinity to the receptor 2.4 times, but does not influence the cAMP production compared to the wild-type hormone, indicating that the efficacy of the hormone receptor complex to stimulate the cyclase dependent G-protein(s) may still be similar. This certainly also shows that it is important to complement receptor binding studies with functional analysis.

Our in vivo studies have shown that the recombinant forms of hPTH are at least as potent as chemically synthesized hPTH

(29% higher doses of the chemically synthesized preparation were used), demonstrating that the yeast and *E. coli* hPTHs were correctly processed and that the molecule folded correctly to give the proper tertiary structure, which is necessary to give full biological activity. Also, QPTH has folding characteristics that make it as active as the natural hormone. However, the reduced receptor binding potency and biological responses of the chemically synthesized hPTH on a molar basis is unexplained, but may be related to an inadequate N-terminal structure that is the

last synthesized part of the peptide and for PTH is of crucial importance for receptor binding and eliciting the biological responses.

ACKNOWLEDGEMENTS

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Expression and Characterization of a Recombinant Human Parathyroid Hormone Secreted by *Escherichia coli* Employing the Staphylococcal Protein A Promoter and Signal Sequence*

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Human parathyroid hormone (hPTH) is a peptide hormone consisting of 84 amino acids (hPTH(1-84)). Employing the promoter and signal sequence of *Staphylococcus aureus*-protein A we have expressed hPTH in *Escherichia coli*. The expressed proteins are excreted to the growth medium, allowing for rapid and easy purification of the desired products. By amino acid sequence analysis and mass spectrometry, we have shown that the major excreted product is correctly processed human identical hPTH(1-84). The purified recombinant hPTH(1-84) stimulates adenylate cyclase activity in rat osteosarcoma cell membranes to exactly the same extent as synthetic parathyroid hormone standards, indicating that the recombinant product has full biological activity.

Human parathyroid hormone (hPTH)¹ is a peptide of 84 amino acids, secreted from the parathyroid gland. The primary translation product is a 115-amino acid preprohormone, and the prepro part is cut off during the secretion process, yielding the 84-amino acid mature hormone (1).

hPTH is a principal homeostatic regulator of blood calcium and phosphate through its actions on kidney and bone (2, 3). At chronically high secretory rates of PTH, causing sustained abnormal concentrations, bone resorption supersedes formation, resulting in the well known pathology of hyperparathyroidism. Prolonged exposure to low/moderate doses of a biologically active PTH fragment stimulates bone formation and has also been reported to be effective in the treatment of osteoporosis by inducing an anabolic response in the bone (4, 5). Thus, hPTH is a molecule of considerable interest regarding both biological and medical aspects. However, so far studies on intact hPTH have been hampered by the limited availability and the high price of the hormone. Hence a system for the efficient expression of hPTH in microorganisms would be very advantageous for the further progression of studies on hPTH and its role in bone biology and disease.

In this report we describe the use of an expression plasmid

where hPTH cDNA is fused to the signal sequence of *Staphylococcus aureus*-protein A, and is under the transcriptional control of the protein A promoter (6). *Escherichia coli* transformed with this plasmid expresses hPTH and secretes several molecular species of hPTH to the periplasmic space and even to the growth medium. As judged by a series of biochemical parameters, we have achieved the expression of hPTH(1-84) as an extracellular peptide in *E. coli*, representing up to 50% of the total amount of PTH-related peptides produced, and mounting to about 1 mg/liter growth medium. The purified product had a correct amino-terminal amino acid sequence, and we could demonstrate full biological activity in an adenylate cyclase assay using rat osteosarcoma cells.

EXPERIMENTAL PROCEDURES

Materials—Restriction enzymes and other DNA-metabolizing enzymes were obtained from New England Biolabs. ¹²⁵I-Antirabbit-IgG was from Amersham Corp., and the NH₂-terminal-specific anti-PTH antibody was bought from CHEMICON. The production and characterization of the other antiserum has been described earlier (7). Synthetic hPTH(1-84) and [Nle⁶,Nle¹⁸,Tyr³⁴]hPTH(1-34)amide were from Sigma.

Bacterial Strains, Plasmids, and Recombinant DNA Methods—*E. coli* strain BJ5183 (8) was obtained from Dr. F. Lacroute (Centre de Génétique Moléculaire du C. N. R. S., Gif-sur-Yvette, France). The cloning of hPTH cDNA has been described elsewhere.²

If not otherwise stated, recombinant DNA methods were performed according to Maniatis *et al.* (9). DNA sequencing was performed on plasmid DNA with Sequenase (United States Biochemical Corporation) according to the suppliers manual. The oligonucleotides used were synthesized with an automated machine (KabiGen AB, Sweden) as described (10).

Cell Growth and Preparation of Cellular Fractions—For testing of PTH-production, *E. coli* was grown in 2 × YT medium (16 g of Bacto Tryptone; 16 g of Bacto Yeast Extract; 10 g of NaCl/liter) containing 0.4% glucose and 0.5 g/liter of ampicillin. Cells were harvested by centrifugation at 10,000 × g for 10 min, and the supernatant was taken as the growth medium fraction. The periplasmic fraction was prepared by the osmotic shock method described by Nossal and Heppel (11). The soluble intracellular fraction was prepared by sonicating the cell pellet remaining after extraction of the periplasmic proteins. The cell pellet was suspended in phosphate-buffered saline, 0.05% Tween 20 and sonicated 5 × 15 s on ice in a model W-10 Sonicator (Ultrasonics). Cell debris was spun down, and the supernatant was used as the soluble intracellular fraction.

Radioimmunoassay—Radioimmunoassay of hPTH was carried out as described (7) using an antiserum reactive against epitopes between amino acids 44 and 68 in hPTH.

Polyacrylamide Gel Electrophoresis and Immunoblotting—Polyacrylamide electrophoresis in the presence of SDS (SDS-PAGE) was performed as described by Laemmli (12). Samples were solubilized in

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¹ The abbreviations used are: human parathyroid hormone, hPTH, PTH, parathyroid hormone; bPTH, bovine PTH; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; SDS, sodium dodecyl sulfate.

² Høgset, A. (1990) *Biochem. Biophys. Res. Commun.* 166, 50-60.

a buffer containing 0.1 M Tris-HCl, pH 7.5, 17% glycerol, 4% SDS, 0.05% bromophenol blue, and 2% β -mercaptoethanol and incubated on a boiling water bath for 5 min before being loaded on a 15% gel. Electrophoresis was run for 2–3 h at 600 V constant voltage.

For electrophoresis in the presence of acetic acid and urea, the gel was made up with a solution containing 4.5 M urea and 0.9 M HAc. Freeze-dried samples were dissolved in a sample buffer containing 0.9 M HAc, 8 M urea, 2% 2-mercaptoethanol and 0.05% pyronin Y. Electrophoresis was run in 0.9 M HAc at 180 V till the dye had migrated close to the end of the gel.

Proteins fractionated by SDS-PAGE were transferred electrophoretically to Immobilon polyvinylidene difluoride transfer membranes (Millipore) using the buffers of Towbin *et al.* (13) and a "Semi-dry Electrophoretic model B" from ANCOS APS. The transfer was complete after 2 h at 0.2 A constant current. Acetic acid-urea gels were soaked three times for 15 min in transfer buffer containing 0.01% SDS, and electroblotted in the same buffer, as described above.

For staining of total proteins the filters were stained in 0.1% Coomassie-R in 50% methanol for 10 min, followed by destaining in 50% methanol, 10% HAc three times for 5 min and air drying of the filters.

For antibody probing the stained filters were soaked in methanol for a few seconds and rinsed in water for 5 min. Unspecific binding sites on the filters were blocked by incubating the filters in phosphate-buffered saline with 5% non-fat dry milk for 1 h at room temperature. Antibody incubations and washes were performed according to Towbin *et al.* (13). Cock anti-PTH antiserum that reacts with epitopes within amino acid number 44–68 (7) was used (dilution 1:8000) as a primary antibody and rabbit anti-cock-IgG (dilution 1:1000) as the secondary antibody. As a tertiary antibody we used 125 I-antirabbit-IgG from donkey. The NH_2 -terminal specific anti-hPTH rabbit antiserum was used (dilution 1:1000) with a secondary 125 I-antirabbit-IgG from donkey. Autoradiography was performed overnight at -70°C with Kodak X-Omat AR5 film and an intensifying screen.

Concentration and Purification of hPTH by S-Sepharose Chromatography—Cultures (0.5 or 1 liter) were grown in 1 or 2-liter Erlenmeyer flasks at 37°C in a shaking incubator. The cultures were grown to an OD_{600} of 2.0–2.8 and harvested by centrifugation. The pH in the supernatant (medium fraction) was adjusted to 2.95 by the addition of HCl, and the acidified medium was filtered through a Whatman GF/C filter. The filtrate (usually 5–10 liter) was applied on a column of S-Sepharose (Pharmacia LKB Biotechnology, Inc. column volume 300 ml) at a flow-rate of 300 ml/min. After application the column was washed by 450 ml of 0.1 M HAc, pH 6.0, and PTH was eluted with 750 ml of 0.1 M Na_2HPO_4 , pH 8.5. The elution of proteins was monitored by reading the absorbance at 280 nm, total PTH in the fractions was detected by radioimmunoassay, and the molecular species were characterized by electrophoresis and immunoblotting. After elution the column was washed with 4 bed volumes of 0.1 M NaOH followed by 0.5 bed volumes of water, and regenerated by 4–5 bed volumes of 0.3 M glycine, pH 3.0.

Purification of PTH by HPLC—PTH from the peak fractions after S-Sepharose chromatography was further purified by reversed-phase HPLC using a Vydac RP C_{18} protein/peptide column. For small samples (up to 5 ml) a 25-cm \times 4.6-mm column was used. Larger samples (5–50 ml) were chromatographed on a 30-cm \times 22-mm column (Vydac, Mojave Hesperia, CA), using LDC constametric pumps model I and III, LDC gradient master, LDC spectromonitor II (LDC, Milton Roy Co, Riviera Beach, FL) and a Vitatron 2 channel recorder. The experimental conditions in the first HPLC purification were as follows. Eluant A, consisted of 0.1% trifluoroacetic acid in filtered and distilled H_2O . Eluant B, consisted of 70% acetonitrile in eluant A. Flow was 1.0 and 10.0 ml/min for small and large columns, respectively. Gradient was 35–55% eluant B (linear) in 48 min. Washing was with 100% B for 10 min and equilibration with 35% B. In the second HPLC purification, the small column was used under the same experimental conditions as in the first HPLC purification with the following exception: gradient was 44–49% B for 40 min.

Amino Acid Sequence Analysis—Amino acid sequencing was either done directly on fractions from the second HPLC purification, or it was performed on proteins separated by SDS-PAGE (14). In this case the proteins were blotted to a polyvinylidene difluoride filter, and the filter was Coomassie-stained as described. Interesting bands were cut out from the filter by a sterile scalpel and sequenced in a 477A protein sequencer with an on-line 120A phenylthiohydantoin amino acid analyzer from Applied Biosystems (Foster City, CA). All reagents were obtained from Applied Biosystems. Amino acid sequencing was

performed by Dr. K. Sletten, Institute of Biochemistry, University of Oslo.

Mass Spectrometry—The molecular mass of purified recombinant hPTH was determined by ^{252}Cf plasma desorption mass spectrometry utilizing a Bio-Ion 20 instrument (Bio-Ion Nordic AB, Uppsala, Sweden). Recombinant hPTH (1 nmol) was dissolved in a 0.1% solution of trifluoroacetic acid in water containing 1 nmol of glutamic acid. Approximately 5 μl of this solution was applied to an aluminum sample foil coated with a thin layer of nitrocellulose and dried under a stream of nitrogen. The sample was analyzed in the mass spectrometer without rinsing the nitrocellulose layer after sample application, since rinsing completely abolished the PTH peaks in the spectrum. An acceleration voltage of 18 kV was used for collection of the spectra. Time-of-flight measurements were made with a resolution of 1 ns and converted to mass spectra using the time centroids for H^+ and Al^{3+} , respectively. Data were collected for 4 h, and the spectra were printed after background subtraction.

Adenylate Cyclase Assay—Recombinant hPTH purified on HPLC and characterized by gel electrophoresis and NH_2 -terminal amino acid sequence analysis was freeze dried. It was dissolved in distilled water and diluted to yield doses capable of stimulating the adenylate cyclase of UMR 106 rat osteosarcoma cell membranes. Cell membranes were prepared and the assay carried out as previously described (15, 16). The experiments were performed in triplicate determinations which differed by less than 17%. hPTH(1–84) and [Nle^8 , Nle^{18} , Tyr^{34}] bPTH(1–34)-amide from Sigma were used as references.

RESULTS

Expression Plasmid Construction—An expression plasmid (pSPTH) was constructed where cDNA for hPTH(1–84) was positioned after DNA coding for the promoter and signal peptide of *S. aureus*-protein A. This plasmid should express a fusion protein consisting of the protein A signal peptide and hPTH(1–84). Thus, hPTH(1–84) could be expected to be translocated to the periplasmic space (6), and the signal peptide should be cleaved off during this process.

The construction of the expression plasmid is outlined in Fig. 1. The *Bgl*III-*Xba*I fragment containing the entire hPTH coding region was excised from the plasmid pSSHPTH10² and inserted between the *Bam*HI and *Xba*I sites in pUC19. This plasmid (designated pUC19PTH) was then cleaved with *Ava*I and *Xba*I and the resulting 300-base pair fragment was inserted between the *Ava*I and *Xba*I sites in pKP43 giving the plasmid pKP43PTH. To get a protein A identical signal peptide correctly positioned in front of the PTH coding sequence, pKP43PTH was cleaved by *Ava*I and *Nsi*I. A synthetic oligonucleotide (see Fig. 1) was then inserted between these sites to give the final expression plasmid pSPTH. The correct sequence of the expression plasmid was confirmed by sequencing of plasmid DNA.

Production of hPTH—Several *E. coli* strains were transformed with the expression plasmid pSPTH, and different cellular fractions were tested for hPTH production by radioimmunoassay. Of the strains tested BJ5183 gave the highest overall level of expression, and this strain was therefore chosen for a more detailed study of hPTH expression.

A time course of the PTH production in this strain transformed with pSPTH is shown in Fig. 2. While the overall production of PTH increased in parallel with the OD_{600} of the culture, the localization of hPTH-immunoreactive material changed as a function of growth time. At early growth stages most of the PTH was located in the periplasmic space, while more than 80% of the total PTH immunoreactivity had accumulated in the growth medium at the stationary phase of growth. It was also apparent that the secretion process must be very efficient in that only a very small fraction of the total hPTH-related material was found in the intracellular fraction.

Analysis of Expression Products by Polyacrylamide Electrophoresis and Immunoblotting—To analyze the expression products in more detail, we subjected proteins from the growth

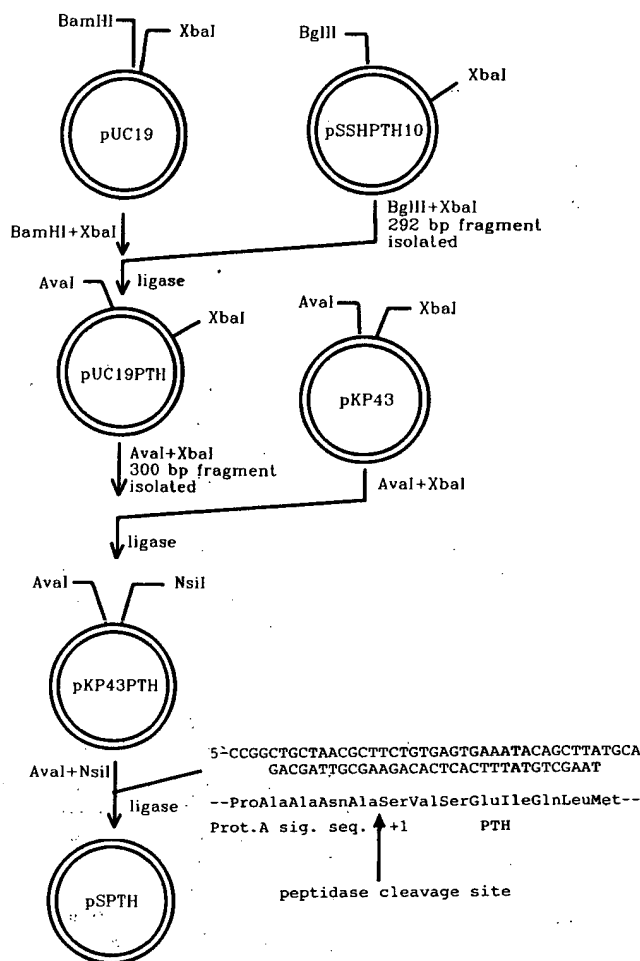


FIG. 1. Construction of the expression plasmid pSPTH. For details, see text.

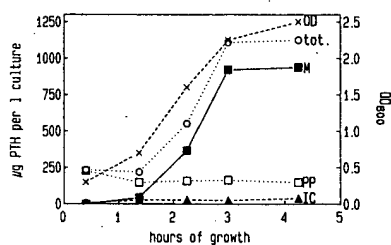


FIG. 2. hPTH production as a function of culture growth. Cells were grown as described, and the amounts of hPTH immunoreactive material in the periplasmic (PP), medium (M), and intracellular (IC) fractions were determined by radioimmunoassay. OD₆₀₀ of the culture (OD) was determined in dilutions to about OD 0.5, and the total amount of PTH immunoreactivity produced is also shown (tot.).

medium and the periplasmic space to SDS-PAGE and immunoblotting.

In Fig. 3 (lane 1) is shown an experiment where proteins from the growth medium have been subjected to immunoblotting using the middle/COOH-terminal antibody. Four major bands can be seen, one 9.5-kDa band comigrating with the PTH standard, two smaller bands with M_r values of about 6,000 and 5,500, and one larger band with an M_r of about 13,500. As discussed below some of these peptides have been purified and subjected to NH₂-terminal amino acid sequence analysis.

As can be seen from Fig. 3 (lane 2), four major hPTH-related peptides could be detected after immunoblotting of

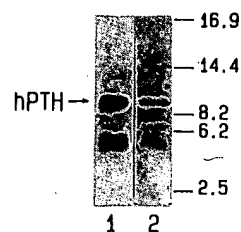


FIG. 3. Analysis of expression products by SDS-PAGE and immunoblotting: comparison of products found in the periplasmic and the medium fractions from *E. coli* transformed with pSPTH. Cells were grown, and the medium and periplasmic fractions were prepared as described. The fractions were concentrated by freeze drying, and amounts representing 100 µl of the culture were applied on the gel (lane 1, medium; lane 2, periplasm). Electrophoresis and immunoblotting were performed with the middle/COOH-terminal specific antibody as described. The positions of hPTH and molecular weight standards are indicated.

the periplasmic proteins. One of these peptides had an M_r of 9500 and comigrated exactly with the hPTH standard.

The largest of the immunoreactive peptides from the periplasm has an M_r of about 14,500 and suggestively represents the signal sequence hPTH fusion protein with an uncleaved signal sequence.

The smaller immunoreactive bands probably represent proteolytic degradation products of hPTH. The major smaller bands from the periplasm have M_r values of about 6500 and 5500, and the smaller of these proteins comigrates with a protein also found in the growth medium. The 14.5-kDa band seen in the periplasmic fraction has never been observed in the growth medium fraction, indicating either that this peptide remain in the periplasm, or that it is cleaved during or after excretion to the medium. The smaller bands might correspond to cleavage of the PTH molecule at about position 25 and 35. These regions are known to be susceptible to proteolysis in other systems (17) and might be acted upon by a variety of proteases. In all experiments the predominant band is the band comigrating with the PTH standard (Fig. 3 is a representative example). However, this band generally seem to constitute a greater proportion of the total immunoreactive material in the growth medium than in the periplasm.

Purification of hPTH Species from the Growth Medium—PTH was concentrated from the growth medium by chromatography on S-Sepharose, and PTH in the fractions was detected as described under "Experimental Procedures" (data not shown). Fractions containing hPTH(1-84) were then subjected to reverse-phase HPLC. As shown in Fig. 4A, a major peak (fractions 32 and 33) with the same retention time as standard hPTH could be identified. Proteins from this and from the other major peak (fraction 16) were freeze-dried and subjected to SDS-PAGE and immunoblotting. As shown in Fig. 5A (lanes 1 and 2), the peak with the same retention time as hPTH mainly consists of two proteins, a main component with an M_r identical to an hPTH standard and a minor component with an M_r of about 13,500. Both of these peptides react with an anti-PTH antibody on immunoblots (Fig. 5B, lanes 5 and 6). The main component (hereafter called recombinant hPTH) was further purified by another round of reverse-phase HPLC as described. As shown in Fig. 4B, this procedure resolved the two components into two peaks. The major peak eluted exactly like an hPTH(1-84) standard (Fig. 4, B and C). Finally, when this peak and standard hPTH(1-84) were cochromatographed one symmetric peak appeared, indicating that the recombinant hPTH behaved exactly as the hPTH standard under these experimental conditions (Fig. 4D). SDS-PAGE of the peak fraction showed one band co-

FIG. 4. Purification of recombinant hPTH from the growth medium of *E. coli* BJ5183. Recombinant hPTH was purified as described under "Experimental Procedures." A, chromatogram (A_{220}) of the first HPLC purification. Fractions used for SDS-PAGE and second HPLC purification are indicated. B, chromatogram of the second HPLC purification of fractions 32 and 33 from panel A. The peak of recombinant hPTH is indicated in black. C, second HPLC run of 1 μ g of standard hPTH(1-84). D, cochromatography of the recombinant PTH peak from panel B and 1 μ g of standard hPTH(1-84).

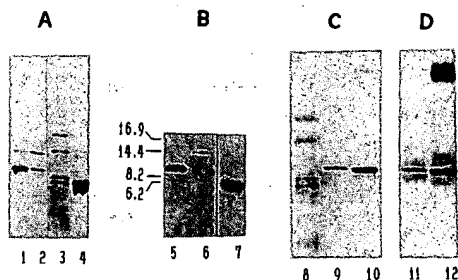
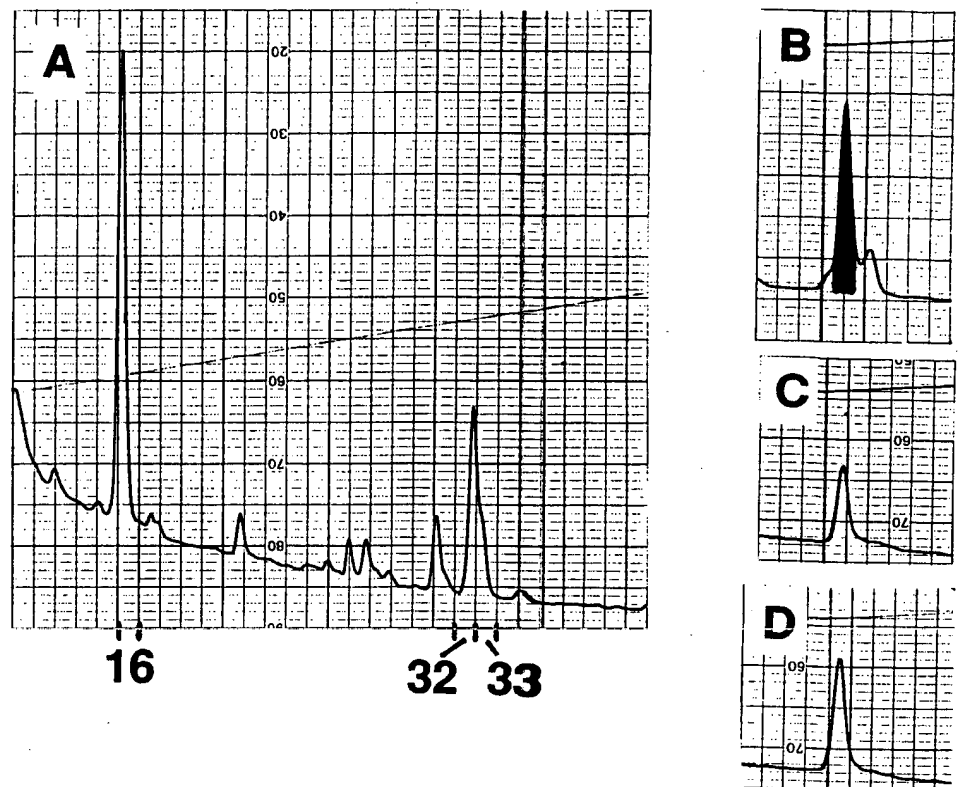


FIG. 5. SDS-PAGE and immunoblotting of peaks from first and second HPLC purification. Proteins from the fractions indicated in Fig. 4 were subjected to SDS-PAGE and immunoblotting as described. Molecular weight standards are indicated. A, Coomassie Brilliant Blue staining of the filter after SDS-PAGE and blotting of fractions from the first HPLC run. B, autoradiogram of an immunoprobe filter (using the middle/COOH-terminal antiserum) of the same samples electrophoresed on a parallel gel (20 times less material was loaded on this gel than on the one shown in panel A). C, Coomassie Brilliant Blue and, D, silver staining of the gel after SDS-PAGE of the proteins in the recombinant hPTH peak indicated in Fig. 4B. Loadings were as follows. 1 and 5, fraction 32; 2 and 6, fraction 33; 3 and 8, molecular weight standards; 4 and 7, fraction 16; 9 and 11, recombinant hPTH peak, 1 μ g; 10 and 12, hPTH(1-84) standard, 3 μ g.

migrating with the hPTH standard (Fig. 5, C and D), suggesting that the recombinant hPTH was essentially pure and that it behaved exactly like the hPTH standard also in this separation system.

The recombinant hPTH from the HPLC purification was then subjected to NH_2 -terminal amino acid sequencing as described, and the result is shown in Table I. We were able to determine unambiguously 45 amino acids from the NH_2 -terminal, and the determined sequence was identical to the known sequence of hPTH (18, 19). The sequence analysis also indicated that the recombinant hPTH was more than 90% pure after the three purification steps employed. The

TABLE I

NH_2 -terminal amino acid sequences of PTH species purified from the growth medium

PTH immunoreactive peptides were purified from the growth medium and subjected to amino acid sequence analysis as described. A, recombinant PTH peak (Fig. 4B) (45 residues from the NH_2 -terminal were sequenced; only the first 29 are shown in this table). B, 13.5-kDa band (Fig. 5A, lane 2). C, 6.0-kDa band (Fig. 5A, lane 4).

A: SVSEIQLMHNLGKHLNSMERVEWLRKKLQ
B: SVSEIQL
C: EWLRKKLQ

repetitive yield in this analysis was 94.5% for Leu in positions 7, 11, 15, 24, 28, and 41, and 94.5% for Val in positions 2, 21, 31, and 35.

To further substantiate the conclusion that the 9.5-kDa protein is intact hPTH(1-84), we performed acetic acid-urea polyacrylamide gel electrophoresis and immunoblotting on proteins from various stages of the purification. In this separation system proteins are separated according to a combination of charge and size. The results of such an experiment are shown in Fig. 6. It can be seen that the main immunoreactive protein in the fraction containing recombinant hPTH comigrates with the PTH standard also in this separation system (lanes 2 and 5).

To show that the purified recombinant hPTH represented the intact hormone, we performed mass spectrometry as described under "Experimental Procedures." The plasma desorption mass spectrum obtained from the recombinant hPTH adsorbed on the sample foil covered with nitrocellulose is shown in Fig. 7. A molecular mass of 9426 ± 9 daltons could be calculated from the single-charged and the double-charged molecular ions present in the spectrum. The theoretical molecular mass of hPTH calculated from the amino acid composition is 9425 daltons, thus corresponding nicely to the value determined for the recombinant hPTH by mass spectrometry.

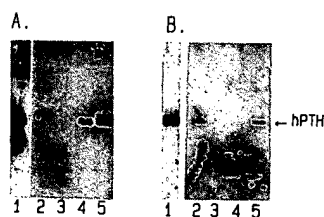


FIG. 6. Acetic acid/urea-PAGE and immunoblotting of expression products. Proteins were fractionated by acetic acid/urea-PAGE and blotted as described. Parallel filters were probed with the middle/COOH-terminal specific antiserum (A) and the NH₂-terminal specific (B) antibody. Loadings were as follows. 1, proteins from the PTH-containing peak after S-Sepharose chromatography. 2, 50 ng of standard hPTH. 3–5, fractions 34, 33, and 32, respectively, from the first hPTH-purification (Fig. 4A).

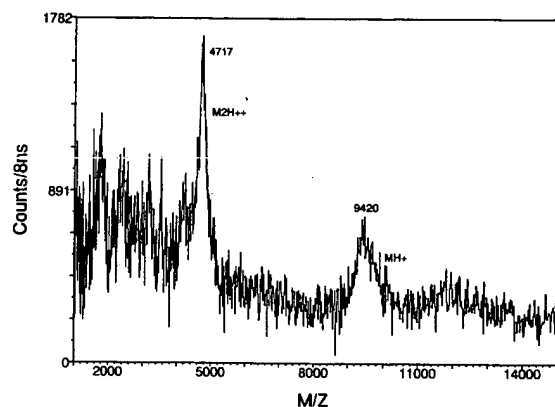


FIG. 7. Plasma desorption mass spectrometry analysis of purified recombinant hPTH(1–84). The spectrum shows the ion abundance as a function of mass over charge. The calculated molecular mass of native PTH is 9425 daltons.

We have also sequenced the corresponding 9.5-kDa protein isolated from the periplasm, and we found that also this protein had an NH₂-terminal amino acid sequence identical to hPTH (data not shown).

The immunoblot analysis of the HPLC-peaks also revealed two other peptides that reacted with the anti-PTH antibody, namely a 6.0-kDa peptide from fraction 16 (Fig. 4A, and Fig. 5, lanes 4 and 7) and the 13.5-kDa minor constituent of the peak containing recombinant hPTH (Fig. 5, lanes 2 and 6). These peptides probably are the same as the three largest peptides detected in immunoblots of unfractionated material from freeze-dried medium shown in Fig. 3 (lane 1). The 6.0-kDa peptide from fraction 16 was cut out from the filter shown in Fig. 5A (lane 4) and subjected to NH₂-terminal amino acid sequence analysis. The amino acid sequence showed that this peptide was a fragment of hPTH, starting at amino acid 22 and probably extending all the way through to the COOH-terminal end of hPTH(1–84) (Table I).

The NH₂-terminal sequence of the 13.5-kDa peptide (Table I) was determined by cutting out the Coomassie-stained band after SDS-PAGE and blotting of proteins from the minor peak from the second HPLC run (Fig. 4B).

As shown in Table I, this peptide has an NH₂-terminal amino acid sequence that is identical to that of hPTH, despite being about 4 kDa larger than hPTH(1–84). At present the molecular identity of this 13.5-kDa peptide is unclear. It is interesting to note, however, that an hPTH-immunoreactive peptide with the same *M_r* is observed also when hPTH is expressed as an intracellular peptide in *E. coli*.² Thus, the production of the 13.5-kDa peptide does not seem to be a consequence of the expression of hPTH as a secreted peptide

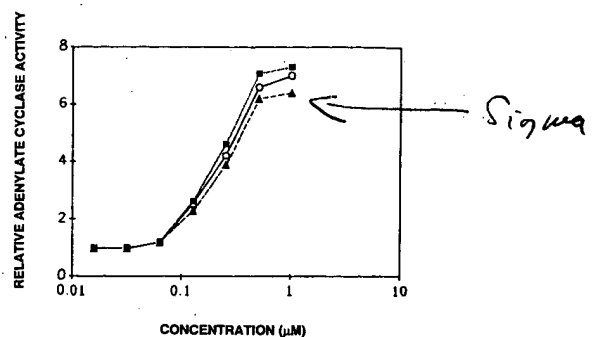


FIG. 8. Adenylate cyclase assay of purified recombinant hPTH. Protein from the recombinant hPTH peak indicated in Fig. 4B was tested in the adenylate cyclase assay as described. The basal adenylate cyclase activity was chosen as 1.0 on the ordinate. The amount of PTH was determined by radioimmunoassay of the same solution as used in the adenylate cyclase assay. (▲---▲) hPTH(1–84) standard, (○---○) recombinant hPTH, (■---■) [Nle⁸, Nle¹⁸, Tyr³⁴]bPTH(1–34)amide standard.

but might possibly be due to a readthrough of the PTH stop codons or to some kind of covalent modification of hPTH introduced inside the *E. coli* cell.

Biological Activity—The purified recombinant hPTH was tested for biological activity in the adenylate cyclase assay as described. It can be seen that the recombinant hPTH stimulated adenylate cyclase to the same extent as the hPTH(1–84) and [Nle⁸, Nle¹⁸, Tyr³⁴]bPTH(1–34)amide standards, indicating that the recombinant hPTH has a specific biological activity that in this assay is indistinguishable from that of the standards (Fig. 8).

DISCUSSION

To our knowledge this work represents the first report on the successful expression and secretion of intact hPTH from *E. coli*. Other investigators have expressed hPTH as an intracellular peptides in *E. coli* (20, 21, 36) or have tried to achieve secretion of hPTH from *E. coli* using hPTH's own signal sequence (22). In the last report three forms of PTH were produced. One of these, hpreproPTH, became associated with the outside of the inner membrane, while the two other forms, hPTH(3–84) and hPTH(8–84), were intracellular peptides. The authors concluded that the hPTH signal sequence is able to confer translocation of hPTH through the inner membrane but that this signal peptide is not cleaved off by the signal peptidase, causing hpreproPTH to remain in the inner membrane.

In contrast, the protein A signal sequence used in the present work seems to be very effective in translocating hPTH through the inner membrane, indicated by the observation that only a few percent of the total hPTH immunoreactivity was located in the intracellular fraction. It also seems that the signal sequence is efficiently cleaved off during secretion. Although molecules probably corresponding to the uncleaved fusion protein could be detected in the periplasmic fraction by immunoblotting, these molecules constituted only a small amount of the total immunoreactive peptides.

A very unexpected finding was that a large amount of the immunoreactive material was excreted to the growth medium. The reason for this is at the moment unclear. It is not, however, due to cell lysis or a general leakage phenomenon because the ratio of hPTH to total protein is much higher (about 15 times, data not shown) in the medium than in the periplasmic fraction. Also the fact that the hPTH species in the medium are partly different from those seen in the periplasmic fraction (see below) argues against a generally unsp-

cific leakage of material from the cell.

Normally *E. coli* excretes very few proteins to the growth medium. The mechanism for excretion is largely unknown, but, at least in some cases, it does not seem to involve consensus signal sequences (23). In a few cases heterologous proteins have, however, been reported to be excreted after expression as fusion products with bacterial signal sequences (24–28). Excretion has also been achieved by making fusions of heterologous proteins and the signal sequence and a modified part of *S. aureus*-protein A (29–31). In this case it seems like the modified protein A part in some way makes the outer membrane leaky, so that periplasmic proteins can escape to the growth medium. The protein A signal sequence in itself does not generally seem to be able to translocate proteins to the growth medium since, for example, a fusion of this signal sequence to alkaline phosphatase expresses alkaline phosphatase as a periplasmic protein only (29). This indicates that hPTH or the fusion protein might have a structure that enables it to pass relatively easily through the outer membrane of *E. coli*, either passively or via some kind of transport system. The processes responsible for the release of intact hPTH to the medium seem to be discriminate, since some of the hPTH species in the growth medium are different from those in the periplasmic fraction and vice versa. For example, is the putative complete 14.5-kDa fusion protein never found in the medium, indicating that this form either is unable to pass through the outer membrane, or that it is in some way processed during or after translocation?

Likewise is the 13.5-kDa hPTH-form not found in the periplasmic space, suggesting that this form is either rapidly degraded in this compartment, or that it is efficiently exported to the growth medium?

The smaller PTH species observed are probably produced during or after secretion, presumably by proteases different from the signal peptidase. Such degradation of secreted proteins in *E. coli* has been described before (32), and the observation that the smaller PTH species could not be observed when PTH is expressed as an intracellular product,² supports the notion that the degradation in some way is associated with the secretory process.

It is at the moment unclear whether the PTH excreted passes through the periplasmic space or if it is excreted directly through the inner and the outer membrane at the same time. The fact that at least a fraction of the excreted PTH is correctly processed (presumably by signal peptidase I, see for example Ref. 33), may however, indicate that at least some of the PTH molecules go through the usual pathway for secretion of proteins to the periplasm before being excreted to the growth medium.

Our approach for producing recombinant hPTH in *E. coli* seems to have several advantages over those previously reported where hPTH has been expressed as an intracellular protein (20, 21, 36). In some of these studies, the production of hPTH was low, partly because intracellular hPTH is rapidly degraded (20, 21). In the present work the hPTH production was 10–50 times higher than what was reported in these studies. The reason for this may be that heterologous proteins tend to be more stable when released to the periplasm which may contain less proteolytic activity than the intracellular compartment (34). Even more favorable conditions may exist for proteins released to the growth medium explaining why intact hPTH accumulates in the medium during growth. Such an accumulation of hPTH could not be seen in the intracellular or periplasmic fractions, indicating a more rapid proteolytic turnover of hPTH in these compartments or escape by secretion.

Wingender *et al.* (36) recently reported on high yields when hPTH was expressed as an intracellular fusion protein. These authors, however, were unable to produce human identical hPTH(1–84), their main product being Pro-hPTH.

Expression of hPTH as a secreted protein also has the advantage of avoiding the problem with the NH₂-terminal formyl-methionine residue necessary for initiation of translation. Although this formyl-methionine residue can be removed from intracellular proteins by a deformylase and a methionine-amino-peptidase, this often is an inefficient process for heterologous proteins especially when these are highly expressed (35). In the case of hPTH, it has been shown that the removal of this residue is incomplete, even at low expression levels (21). When hPTH is expressed as a secreted protein the NH₂-terminal part of the fusion protein is cleaved off by the signal peptidase, and as long as this cleavage occurs at the right position, the NH₂-terminal of the heterologous protein should be correct. A correct NH₂-terminal is utterly important for hPTH because the biological activity of this hormone is critically dependent on the amino-terminal sequence. For example will the addition or deletion of only 1 amino acid residue usually destroy most of the biological activity (2, 3), although it very recently has been shown that Pro-hPTH has full biological activity (36)? In our expression system the hPTH fusion protein is at least partly correctly processed to hPTH(1–84) during secretion to the growth medium. In addition to hPTH(1–84), however, several hPTH fragments are produced, indicating a certain level of degradation during the secretion process.

Another obvious advantage of our expression system is the ease by which the recombinant hPTH can be purified. As can be seen from Figs. 4 and 5, the recombinant hPTH can be judged to be more than 80% pure after just two purification steps, and the sequence analysis indicates that it is more than 90% pure after the third purification step. This is in contrast to the lengthy purification procedure employed by Rabbani *et al.* (21) to purify recombinant hPTH expressed as an intracellular peptide in *E. coli*.

In conclusion we have succeeded in expressing and purifying recombinant hPTH in *E. coli*. Since the purified 9.5-kDa protein comigrates with an hPTH(1–84) standard in three different separation systems, has the right NH₂-terminal amino acid sequence, and has a correct molecular mass as determined by mass spectrometry, it seems reasonable to conclude that this protein represents intact hPTH(1–84) produced and excreted in *E. coli*. Furthermore, the purified product has full biological activity as determined in the adenylate cyclase assay. In comparison to other published systems for the expression of hPTH in microorganisms, our system gives higher production and easier purification, since the product is excreted to the growth medium. By using this expression system combined with high density fermentation methods, it should now be possible to produce pure hPTH(1–84) in such quantities that physiological and clinical studies using the intact hormone could be performed on a larger scale.

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